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Sugar economy in diapausing pupae of *Celastrina sugitanii* (Lepidoptera, Lycaenidae), with notes on their cold tolerance*

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Abstract Almost all of pupae of *Celastrina sugitanii* entered long diapause for ten months, after short larval stage for *ca* 25 days. Seasonal changes in carbohydrate contents of the pre-diapausing and diapausing pupae were pursued using mainly gas chromatography. Of 6.64 mg/pupa carbohydrates accumulated in their larval period, *ca* 70% was consumed within pre-diapause phase, two weeks after pupation, at a rate of 0.32 mg/pupa/day, when adult morphogenesis progressed in the pupa. Consumption rate of carbohydrates was then strongly suppressed down to 0.004 mg/pupa/day in diapause phase, despite of higher ambient temperature of 24°C. Trehalose began to accumulate during the pre-diapausing morphogenesis period, increased up to 1.35% of body weight until June 20. Both glucose and inositol contents decreased concomitantly with the trehalose production. The trehalose content was stable throughout diapause phase irrespective of exposure to chilling stimuli at 5°C. Although two *Celastrina* species, *C. sugitanii* and *C. argiolus*, can be regarded as trehalose accumulating type in terms of cryobiology, their supercoolig points were different from each other: The pupae of *C. sugitanii* supercooled down to –15°C probably without freezing tolerant ability, while the pupae of *C. argiolus* supercooled down to –22.4°C.

Key words *Celastrina sugitanii*, carbohydrate consumption, adult morphogenesis, diapause, supercooling.

Introduction

So-called "spring ephemerals", species in the temperate zone of which adults appear only in early spring, have to pass long diapausing period under regime of seasonal changes there (Ishii, 1988). Their larval period is restricted, usually several weeks. This implies that in such species, almost all of assimilation is performed within the short period, and substances produced by the metabolism have to support almost activities in the rest of life cycle, for a long duration in physical time. To maximize efficiency in utilization of substances assimilated, therefore, should be of crucial significance on evolution of life cycle traits as "spring ephemerals".

As a representative of such species, we pursued changes in carbohydrate contents of diapausing pupae of *Celastrina sugitanii* Matsumura, which has a univoltine life cycle in Japan. Although some geographic variations in biology were hitherto known in the species (Fukuda *et al.*, 1984), in a wide area, the adults appear late April to early May and the larvae are specialist depending on flower of *Aesculus turbinata*. Annual energy intake of the species is hence performed mostly within very short flowering period of the host tree, during a few ten days at most (Komeyama and Hoshikawa, in press). In the present paper, we report process of carbohydrate consumption and inter-conversion among relevant substances in the pupae of *C. sugitanii*, in both pre-diapause and diapause phases. Adult morphogenesis in the pupa, formation of pharate adult, and hibernating strategy of the butterfly

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*Life cycle of the genus *Celastrina* in Japan, I.

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are also described briefly herewith, comparing with those of a congeneric multivoltine species, *C. argiolus* (Linnaeus).

Materials and methods

Materials

About 80 mature larvae were collected by "litter-fall traps" set under a host-tree, *Aesculus turbinata*, in late May 2004, at Misasa, near Kurayoshi City, Tottori Prefecture (Komeyama and Hoshikawa, in press). Until analyses mentioned below, pupae obtained from the larvae were reared under room temperature (24–27°C) until June 20, at 24°C until November 11, and transferred to 15°C for acclimation, then to 5°C on November 16; kept in dark except observation time. Fifty pupae were subjected to measurement of supercooling point and/or carbohydrate analyses, and 10 were dissected to observe process of adult morphogenesis under a binocular microscope. About 15 pupae died during the experiment probably due to desiccation, in which at least 2 individuals developed without diapause, as complete adults with colored wings were found in the pupae dissected in August. The remained 6 pupae were incubated at higher temperatures (15–21°C; 16L8D) for adult emergence since March 17, 2005. As a reference, 10 diapausing pupae of *C. argiolus*, which reared at 18°C (8L16D) since egg stage, were jointed to the above 5°C stock of *C. sugitanii* on December 2, and subjected to the same analyses.

Measurement of supercooling point

The pupa was cooled in a box with a block of dry ice. The pupa was covered double with vinyl tube and glass tube to reduce cooling rate and its temperature was monitored by a copper-constantan thermocouple laid surface of pupa. In a cooling curve thus obtained, the temperature at which turned to rise by releasing latent heat due to freezing was defined as supercooling point. Cooling rate was *ca* 0.5°C/min under the condition. Most pupae frozen were analyzed successively their carbohydrate contents, while 9 pupae frozen on January 9 were kept at 5°C after thawing, and incubated by the same treatment with the above intact 6 pupae, to examine their freezing tolerant ability.

Measurement of carbohydrate contents

After measuring the body weight, each pupa was homogenized with 2 m*l* of 80% ethanol and 0.03 m*l* of mesoerythritol solution (100 mg/10 m*l*) as an internal standard. After centrifuging at 3000 g for 10 min, the supernatant was used for sugar determination and the sediment for glycogen determination. The supernatant was evaporated to dryness under vacuum, to the residue 0.1 m*l* of TMSI-C (trimethylsilylating reagent, Gasukuro Kogyo Co.) was added, and heated at 65°C for 45 min. The resulting TMS-derivative was applied to a gas chromatogram (Shimadzu, GC-4CMPF) using glass column (3 mm × 3 m) with 1.5% OV-1 on Chromosorb W. The temperature was programmed from 130 to 270°C at 5°C/min and held at 270°C for 15 min. The elution profile was followed using a flame ionization detector. As reference aliquots of authentic sugar or sugar-alcohol solutions together with the internal standard were subjected to the same process for trimethylsilylation and gas chromatography. For glycogen determination, the above sediment was washed twice with 2 m*l* 80% ethanol and suspended with 2 m*l* 10% (w/v) trichloroacetic acid. The suspension was boiled at 100°C for 15 min, and centrifuged at 3000 g for 10 min. The glycogen content in the aliquot of the supernatant was determined by the anthrone/sulphuric acid method (Trevelyan and Harrison, 1952; *cf.* Hoshikawa, 1987), in which absorptions at 620 nm were measured by a photometer (Shimadzu UV-1200).

Results and discussion

1. Adult morphogenesis

The pupa of *C. sugitanii* was transparent pink dorsally and transparent green ventrally in color just after pupation, while became opaque brown monotonously until 3-day age. Eyes and apical part of antennae were pigmented since 9-day age and 12-day age, respectively, which could be observed externally. By dissection of pupae each in 0-, 3-, 6-, 9-, 12-, or 15-day age, wings were recognized as transparent membranes in 3-day aged pupa, with

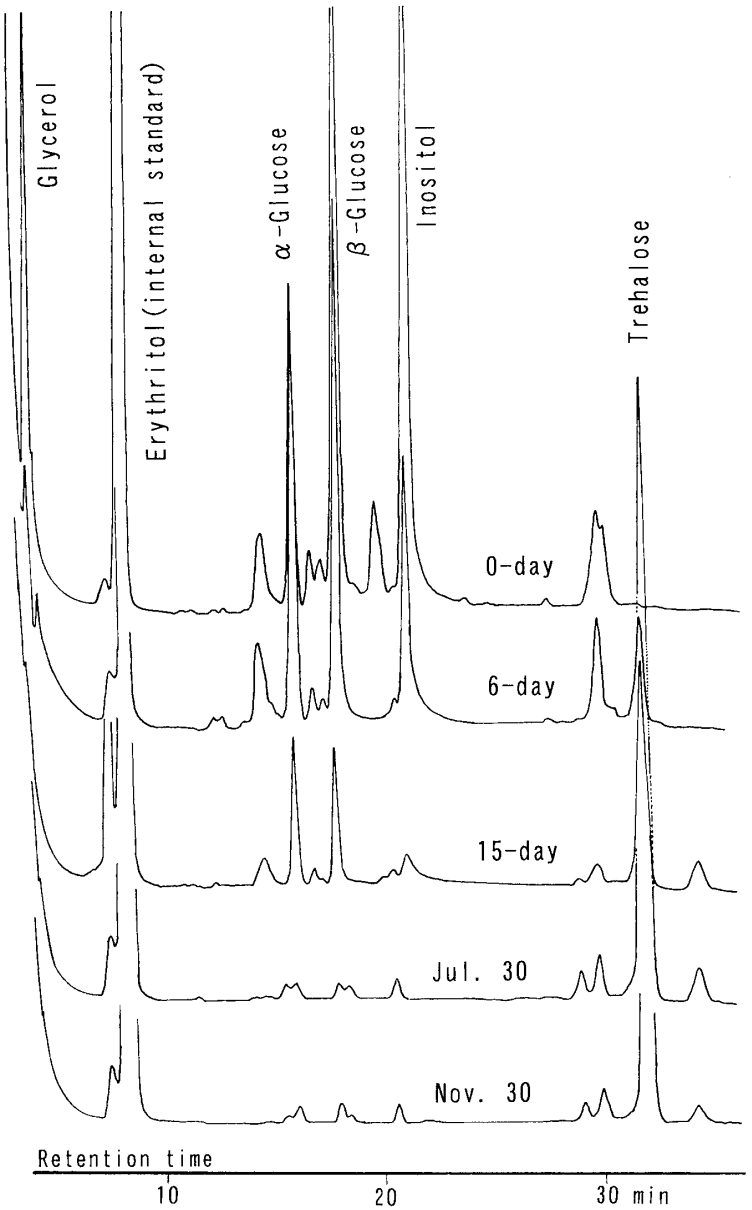

Fig. 1. Representative gas chromatograms of trimethylsilyl derivatives from the extract of the pupae of *Celastrina sugitanii*. During two weeks after pupation, both glucose and inositol decrease, while trehalose starts to increase (0-, 6-, and 15-day aged pupa). Throughout diapause period, the pupae keep to contain a large amount of trehalose (July and November). Small peaks at retention time of 40.3 min are omitted from the figure. Further explanations in the text.

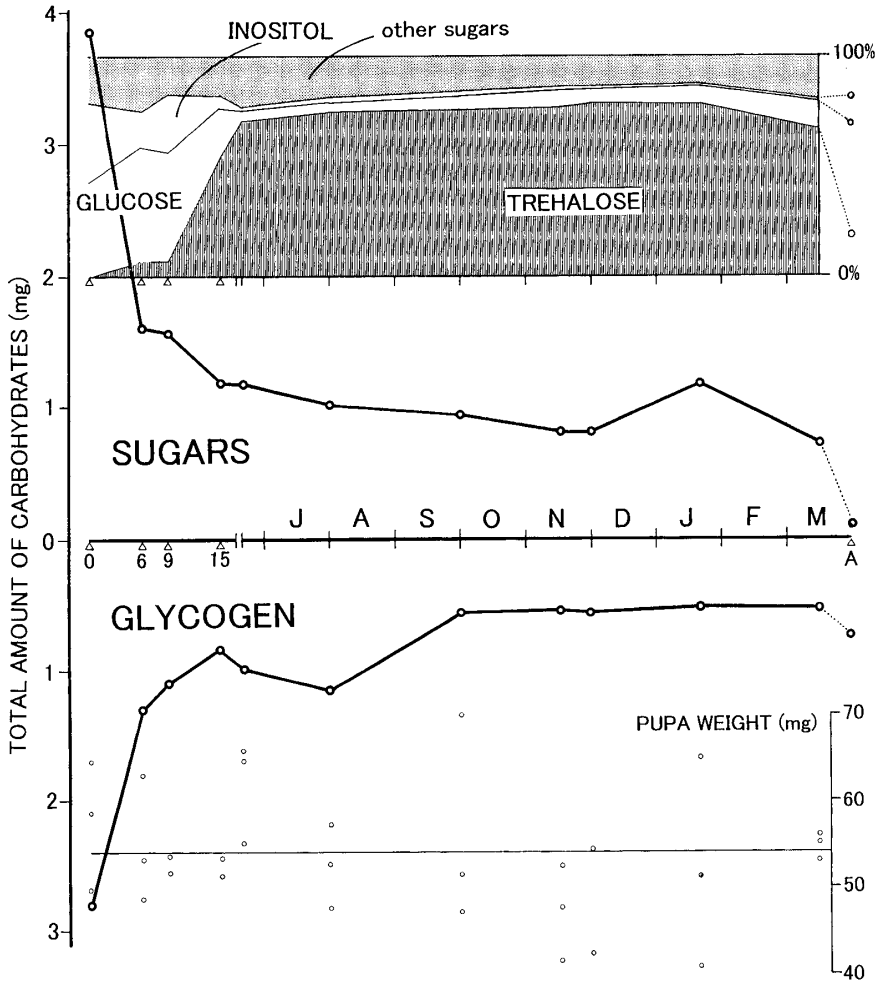

Fig. 2. Seasonal changes in the amount of carbohydrates (sugars+sugar alcohols, and glycogen; mg/pupa) in the pupae of *Celastrina sugitanii* (middle), with sugar components (%; above) and fresh body weight of the pupae analyzed (mg; below). Note abscissa with different intervals; wider 4 times where numerals indicating number of days after pupation. Carbohydrate amount and sugar components of an emerged adult are added at right (A).

whitish veins in 9-day, whole shape of wings were formed in 12-day, which became pinkish milky white glimmering with blue pearl in 15-day aged pupa. Whitish eyes were observed and abdominal spiracles blacken in 6-day. A vacant space was formed at ventral side of basal abdomen since 3-day age and many fat drops were observed there in 3-day aged pupa (the drops were disappeared in 6-day and after). No distinct changes were observed after 15-day age in morphology until the time before emergence. Associated with the morphogenesis, wax coat surrounding pupa thickened in this period presumably due to secretions. The wax coat, which should function as waterproof, was kept thick and hard throughout diapause phase, but cracked finely before emergence, in March. These processes were similar also in diapausing pupae of *C. argiolus*, eyes and apical part of antennae were pigmented since 10- to 12-day aged pupa, but wax coat was very thin in this species. Almost all of pharate adult morphogenesis thus progressed within 15 days after pupation in the both *Celastrina* species.

2. Changes in carbohydrate contents

Some representative gas chromatograms are given in Fig. 1, and reveal 4 major peaks in the

Table 1. Seasonal variations in supercooling points (SCP), sugar contents, and glycogen contents in the pupae of *Celastrina sugitanii*.

Date	SCP (°C)	Weight (mg)	Contents (% body weight)				
	mean±sd (*n*)	mean (*n*)	glucose	inositol	trehalose	glycogen	Total**
0 day*		57.6 (3)	2.92	2.38	0.00	4.91	11.67
6 days*		54.9 (3)	1.53	0.47	0.22	2.34	5.31
9 days*		52.6 (2)	1.49	0.80	0.18	2.07	5.06
15 days*		52.3 (2)	0.45	0.12	1.09	1.59	3.61
JUN 20		62.0 (3)	0.09	0.02	1.35	1.59	3.51
JUL 30		52.5 (3)	0.09	0.05	1.48	2.15	4.13
AUG 31	−15.0±0.3 (3)						
SEPT 30	−14.6±0.4 (3)	56.2 (3)	0.12	0.02	1.30	0.98	2.70
NOV 16	−15.1±0.6 (2)	47.2 (3)	0.14	0.03	1.35	1.14	2.90
NOV 30	−14.5±0.8 (2)	48.3 (2)	0.09	0.03	1.33	1.14	2.83
JAN 9	−15.3±0.4 (9)						
JAN 20		52.3 (3)	0.18	0.02	1.79	1.02	3.30
MAR 15	−15.2±0.3 (6)	54.7 (3)	0.16	0.02	0.89	0.94	2.27
Adult***		16.5 (1)	0.32	0.08	0.12	4.33	4.97
MAR 15#	−22.4±0.7 (5)	40.9 (6)	0.34	0.03	1.64	1.20	3.64
Adult# ***		12.3 (3)	0.34	0.12	0.08	4.80	5.55

*number of days after pupation. "15 days" approximately correspond to June 15.
**including other sugars with retention times similar to fructose and sucrose.
***adult just after emergence was subjected to analysis after removing wings.
#*C. argiolus* for reference. Adults were treated the same as in adult *C. sugitanii*.

pupa of *C. sugitanii*. These peaks were identified by retention times, as glucose for peaks with retention time of 16.6 and 18.4 min (relative ratio of amount/peak area, f=1.46), as inositol for 21.1 min (f=1.18), and trehalose for 32.4 min (f=1.89). Minor components were regarded as glycerol for Rt=4.9 min (f=0.76), probably fructose for Rt=15.2/17.4 min (f=1.40), and probably sucrose for Rt=29.5/30.4 (f=1.76). As for two unidentified minor peaks (Rt=34.7/40.3), their f-value was hypothesized to be 1.90. Figure 2 and Table 1 show seasonal changes in amount (mg/pupa) or contents (% of body weight) of sugars, sugar alcohols, and glycogen in the pupae of *C. sugitanii*, in which sugars on January 20 and glycogen on July 30 might be overestimated. The fresh body weight of the pupae was varied considerably, 53.9±7.3 mg in average with standard deviation, and ranged from 41.5 to 69.9 mg (Fig. 2, below).

2-1. Decrease of carbohydrates associated with adult morphogenesis

Zero-day aged pupae (just after pupation) contained much amount of carbohydrates, 6.64 mg/pupa (Fig. 2), that is 11.7% of fresh body weight of the pupa (Table 1). This amount could indicate a net assimilation of carbohydrates by the feeding larva. The carbohydrates decreased rapidly within two weeks, during the period of adult morphogenesis above mentioned (pre-diapause phase), down to 1.88 mg/pupa (3.6%) in 15-day aged pupae. A discrepancy of 4.76 mg between them shows that *ca* 70% of the carbohydrates were consumed as metabolic fuel during the phase, at a rate of 0.32 mg/pupa/day. The decrease was steeper, 0.63 mg/pupa/day, in the first 6 days (Fig. 2).

2-2. Suppressed respiration during diapause

In contrast to the pre-diapause phase, respiration of pupa was suppressed strongly in the following diapause phase (Fig. 2). More than half of the carbohydrates at the beginning of diapause (2.17 mg/pupa; June 20) maintained until the end of diapause (1.24 mg/pupa; March 15). A regression of the amount of carbohydrates by number of days from June 20 was cal-

culated as, CH mg=2.09–0.0033 days (R^2=0.66, significant at 95% confidence limit). Another regression after removing an overestimated value on January 20 gave a more sufficient result, CH mg=2.12–0.0040 days (R^2=0.89, siginificant at 99%). Thus, the diapausing pupae of *C. sugitanii* consumed carbohydrates at a rate of 0.004 mg/pupa/day, only one-eightieth rate from the rapid consumption in pre-diapause phase aforementioned.

It has been well documented that the onset of diapause is invariably associated with a striking fall in the level of metabolism (Tauber *et al.*, 1986; Danks, 1987; Masaki, 1980). A common ladybeetle *Coccinella septempunctata* for instance, its respiration rate is 2–3 $\mu l O_2$/mg/hr in active season, while reduces to a half, 0.4–1.3$\mu l O_2$/mg/hr in aestivation diapause (determined at 30°C; Sakurai, 1969). So far as carbohydrate consumption concerned, the present results reveal a far lower level of metabolism in diapause, which should reflect the difference in duration of diapause involved. As for another major metabolic fuel, triasylglycerol (=fat), though we can not determined it unfortunately, the observation that fat drops which once appeared in 3-day aged pupa disappeared after 6-day (Result 1) might indicate that most of this substance consumed in pre-diapause phase, probably to produce wax coat surrounding the pupa. During preparation of samples for carbohydrate determination, we did not find any lipid-layer in vials after centrifuging with homogenates of diapausing pupae.

2-3. Trehalose production

Simultaneously with the above consumption, carbohydrate components also altered (Fig. 2 above). Gas chromatograms in Fig. 1 show how large changes in pre-diapause phase whereas how small changes in diapause phase. Trehalose content increased before beginning of diapause, rose from 0% in 0-day aged pupae to 1.35% on June 20 (Table 1). Most of the trehalose production occurred in *ca* 10 days, a period between 9-day and June 20, when decrease of carbohydrates became relaxant. After attaining the plateau on June 20, the trehalose content kept the high level throughout diapause phase, occupied more than 70% of sugars (Fig. 2 above). Despite that the pupae were transferred to 5°C on November 16, no effect of the chilling was observed in the amount of trehalose. Both glucose and inositol contents decreased concomitantly with the trehalose increase. Within the above *ca* 10 days period, decreasing amount of glucose (0.73 mg/pupa; from 0.79 to 0.06) was approximately equal to increasing amount of trehalose (0.74 mg/pupa; from 0.10 to 0.84). Although inositol also decreased in the period (0.41 mg/pupa; from 0.42 to 0.01), however, the substance decreased more in the earliest stage; 1.34 mg/pupa in 0-day age reduced to 0.25 mg/pupa in 6-day age. The function of the inositol accumulated in young pupae is open to future studies. From these changes, most, if not all, trehalose could be derived from glucose.

This species is thus regarded as trehalose-accumulating type in terms of cryobiology. It should be noted that the trehalose accumulation occurred before diapause, in the latter half of morphogenesis period. Although accumulation of cryoprotectants so far known occurs at the beginning of diapause and/or after induced by chilling stimuli (Tsumuki, 1988), the present species might be neither the cases. The earlier cryoprotectant accumulation in *C. sugitanii* may be related with the very low metabolism in diapause phase. Amount of carbohydrates in an emerged adult is additionally given in the right edge of Fig. 2, indicating glycogenesis after diapause together with relative increases of glucose and inositol contents (see also Table 1). Carbohydrate contents of another *Celastrina* species, *C. argiolus*, were similar to those of *C. sugitanii* (Table 1).

3. Change in supercooling points and cold tolerance

As shown in Table 1, pupae of *C. sugitanii* supercooled down to –15.0±0.3°C (*n*=25) without virtual seasonal variation, while pupae of *C. argiolus* down to –22.4±0.7°C (*n*=5), showing a significant difference within the genus. Although supercooling point tends to differ among species hibernating in different microhabitats (Hoshikawa *et al.*, 1988; Leather *et al.*, 1993), the both species hibernate at similar sites (Fukuda *et al.*, 1984). The obvious difference in supercooling point between them may reflect difference in their global distribution (hence also their provenance): the former distributed around Japan Sea, while the latter has wide distribution ranges in Palaearctic and Nearctic Regions (Fukuda *et al.*, 1984). Referring a list of cold tolerance in 109 species of Japanese insects compiled by Asahina (1991), in which 52 lepidopterans including 16 butterflies are listed, cold tolerance of *C. sugitanii* may somewhat similar to that in *Luehdorfia japonica*, while *C. argiolus* to *Araschnia levana*. Since the pupae of both *Celastrina* species repelled water, freezing by ice inoculation should hardly occur.

Hibernating pupae of *C. sugitanii* might be freezing susceptible as all 9 frozen pupae were died. The corpses were seriously damaged, and suggested earlier death of these individuals. This is not conclusive, however, because a high mortality of 5/6 was also observed in the controls, intact pupae reared under the same condition. Due to these pupae were reared at higher temperature of 24°C for a long duration until November 11 in the present study, their diapause development should be arrested, and probably induced a significant prolongation of low-temperature requiring period, extending to longer than 4 months. In hibernating eggs of *Bombyx mori*, it is well evidenced that the period prolongs when cooling treatment is late (Kai *et al.*, 1982; Kai, 1988).

The present results could be outlined as follows: To emerge early spring, "spring ephemerals" have to hibernate as pharate adult. The adult morphogenesis was, however, very expensive for pupa of *C. sugitanii*, which had limited carbohydrates 6.64 mg (100%) accumulated in short larval stage. The insect had to pay so much as 4.76 mg (*ca* 70%) of carbohydrates for the morphogenesis, though it may include cost for wax coat. Since a certain carbohydrates (0.83 mg; *ca* 12%) should be indispensable for the emerged adult, the pupa economized metabolic fuel during long diapausing period, extremely down to 1.2 mg carbohydrate/300 days (*ca* 18%). Under such severe economic circumstances, development of new expensive adaptations, *e.g.* production of more cryoprotectant to lower supercooling point, may be difficult for the species. Such situation should be common, more or less, in most species which pass life cycle as "spring ephemerals".

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摘　　要

スギタニルリシジミ休眠蛹の糖代謝と耐寒性 (星川和夫・米山沙希)

本種は年一化性で10ヶ月以上の期間を蛹 (蛹内成虫) で過ごすので, ほとんど全ての一次同化は約4週間の幼虫期間に行われることになる. 蛹期間における炭水化物 (糖, 糖アルコール, グリコゲン) の消費をガスクロマトグラフィ等により測定した. 蛹あたりの炭水化物含有量は蛹化直後には6.64 mg (生体重の11.6%) であったが, その後2週間の成虫体形成期に約70%が消費され, 休眠開始時は2.17 mg (4.13%) となった. 休眠期の炭水化物消費は成虫体形成期の80分の1以下の速度に抑制され, 9ヶ月後の羽化直前の蛹でも1.24 mg (2.27%) の炭水化物を含有していた.

成虫体形成期にはグリコゲンの他, グルコース, イノシトールも減少し, 一方, トレハロースは体重の1.35%まで蓄積した. 蛹はこのトレハロース含有量を維持したまま越冬するので, 本種はトレハロース蓄積型とみなされるが, その蓄積が休眠開始前であったことは注目される. 越冬蛹は厚いワックス層に覆われ, その過冷却点は–15.0°Cであった.

比較のために分析した多化性のルリシジミの越冬蛹もトレハロース蓄積型であったが, そのワックス層ははるかに薄く, 過冷却点は–22.4°Cであった.

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